International Journal of Agricultural
Science and Research (IJASR)
ISSN(P): 2250-0057; ISSN(E): 2321-0087
Vol. 8, Issue 3 Jun 2018, 147-152
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MICROSPORE CULTURE EFFECTS ON DOUBLE HAPLOID PRODUCTION IN LENTIL (LENS CULINARIS MEDIK)

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ABSTRACT

Establishment of an effective and reproducible protocol is one of the basic prerequisites for the improvement of any crop. Double-Haploid technology has proven to be breakthrough in many crop species and results in the production of homozygous plants in a single step. In spite of a number of success reports of plant tissue culture, technology, very few satisfying and reproducible protocols were available in legumes like a lentil. This study, carried out forthe production of DH plants in the different medium, with various hormone treatments in order to establish a reproducible protocol for microspore regeneration of the cultivar HUL-57. For this determination, the effects of 2 different mediums with 2 hormones on microspore of lentil either through callus induction or directly regeneration were studied. The experiment was done with LS and N6 medium in presence of BAP and 2,4-D results in the non-significant induction of callus from microspores. From the current experiment, this is found that either of the media in various hormones (BAP and 2,4-D) concentrations are non-responsive and became difficult even after combination of different stresses like cold and centrifugation. These reports of non-responsive behavior will be pointing towards trying different medium with the same objective to get the completely homozygous plants.

KEYWORDS: Callus, Hormones, Regeneration & Lentil (Lens culinarisMedik)

ABBREVIATIONS: DH – Double Haploid; BAP – N6-benzylaminopurine; 2,4-D – 2 & 4-dichlorophenoxyacetic Acid

Received: May 01, 2017; **Accepted**: May 22, 2018; **Published**: Jun 18, 2018; **Paper Id.**: IJASRJUN201816

Original Article

INTRODUCTION

Lentil (*Lens culinaris*Medik.) is an important pulse crop and its production takes place in cool season which grown all over the world. Lentil crop seeds have very much nutritive value, especially in developing countries and, their protein content is 20–36% more compared with 8–12% for cereals (Christou, 1993. For plant breeding and molecular genetics programs, double haploid are very important tools. Haploids and DH can be produced by various methods such as apogamy, wide crosses and androgenesis. Conventional breeding methods are not only time consuming, but also not feasible because fully homozygosity is not retained in haploid produced. On the other hand, the recalcitrant nature of legumes species towards DH production is also a great problem. However, regeneration of haploid through induction of androgenesis in some legume species was reported.

To produce embryos through the androgenesis pathway, proper induction of cell division and differentiation is modulated by many factors like, genotype, growth condition of donor, pre-treatment of flower buds, and stages of microspore culture. Along with that, stress pre-treatment also important for proper induction like centrifugation treatment, cold treatment, and osmotic shocks shown a positive effect on induction of haploids. Within legume species, several haploid plants from isolated microspore have been reported in few species like

pigeon pea (*Cajanuscajan* L.) (Kaur and Bhalla 1998), field pea (*Pisumsativum* L.) and grasspea (*Lathyussativus* L.)(Ochatt*et al.* 2009) and more recently in chickpea (*Cicerarietinum* L.)(Grewel*et al.* 2009).

A cold temperature pre-treatment of the immature flower buds prior to microspore isolation was reported important in microspore embryogenesis (Kaur and Bhalla 1998), and recently specific cold pre-treatment at 4^{o}C for 72 hours enhanced induction of androgenesis in chickpea anthers (Grewal*et al.*2009). Centrifugation as an extra stress treatment for induction of androgenesis in legume species which showed beneficial effects in chickpea (Altaf and Ahmad 1986 ; Grewal*et al*. 2009), and in lupin microspores (Campos-Andrada*et al*., 2001; Baylis*et al*., 2004).

Keeping in view of the above facts, the aim of the present study is to develop an efficient protocol for successful induction of haploid from microspore culture of lentil. Lentil is the least exploited species in term of DH production. Keller and Ferrie, (2002) reported calli induction, but not able to regenerate plantlets from that call us. In another study by Croser and Lulsdorf (2004) reports shows that microspores of CDC Crimson and CDC Robin not able to regenerate embryos.

The final objective of this research was to identify the effects of different medium, in various hormone concentrations to produce double-haploid from isolated microspores of lentil.

MATERIALS AND METHODS

The experiment was conducted during the *rabi* season 2016-2017to find strategies for developing Double-Haploid of lentil through microspore culture techniques in different culture media in variety of hormone combinations at the laboratory of institute of agricultural sciences, Banaras Hindu University. The experimental site is located at is situated 25^{0}15' North Latitude and 60^{0}03' East Longitude with an altitude of 128.93 m above sea level.

Plant Material and Growth Conditions

The lentil cultivar HUL-57 was used to experiment. The lentil variety was grown in pots at the farmhouse of institute of agricultural sciences, Banaras Hindu University. Mature seeds were washed using Tween80 (0.5mL/L) for 5 minutes before sterilization. Seeds were surface-sterilized by immersion in 70 % ethanol for 5 minutes, followed by 3 rinses in sterile distilled water. Lentil seeds are grown in plastic pots under favorable conditions. During the vegetative growth of crop plants, adequate supply of water and nutrients are given to avoid any stress which halts plant growth and development. After successful completion of vegetative phase, crop transitions to reproductive phase.

Donor Plants, Genotype, Bud Size and Microspore Stage

Donor plants grown in a favorable environment, without any stress, are a necessity for an androgenic response. Bud size and microspore stage are also important and mainly uninucleate microspores with their high auxin content are used for androgenesis in lentil.

Low Temperature Stress Treatment

Lower temperatures not only increase the length of testing time, but also helpful in obtaining callus and heart-shaped-stage embryos. Buds were directly isolated and pre-treated at 4^{0}C for 72 hours. After the stress, buds put in a 100ml beaker and surface sterilized by stirring in 70 % ethanol for 5 minutes. Buds are transferred to a sterilize mother and microspores were released by squashing buds with a pestle. The resulting microspores were passed through a cheesecloth

filter. Microspore were transferred to centrifuge tube which contains 2,4-D solution (0.1 mg/l). Centrifuge for 3 minutes at 5000 RPM. After that, the supernatant is discarded and microspore was used for inoculation in N6 and LS medium.

Culture Media

Two types of culture medium were used (CHU (N6) (Table 1) and LS (Table 2)) for double haploid production and their Culture medium composition with different hormones (BAP and 2,4-D) summarized (Table 3).

Hormone Preparation

BAP and 2,4-D hormones are used in three different combinations with the medium LS and N6. The hormone concentrations used in the experiment are 0.1 mg/l and 0.5 mg/l in different combination with both the medium which are tabulated in table 3. Each combination is replicated three times with both the medium used.

Table 1: Growth Regulator Composition of Culture Media used in Callus Induction and Regeneration

Medium	Hormone (mg/1)
A1	0.5 2,4-D + 0.5 BAP
A2	0.5 2,4-D + 0.1 BAP
A3	0.5 BAP + 0.1 2,4-D
B1	0.5 2,4-D + 0.5 BAP
B2	0.5 2,4-D + 0.1 BAP
B3	0.5 BAP + 0.1 2,4-D

Two types of media used, A1, A2 and A3 contains LS medium and

B1, B2, and B3 contains CHU (N6) medium with (Bavistin and Amphicllin)

(30mg/l)) 3% (w/v) sucrose and 0.8% (w/v) agar.

RESULTS AND CONCLUSIONS

Culture Medium and Callus Induction

Lentil microspores were cultured on CHU (N6) and LS media with various concentrations of 2, 4-D and BAP hormones are used for production of Double-haploid but the result shows that there was no significant difference with respect to the effect of various hormonal treatments and different types of media. In some of the treatments, it seems to form some callus like projections, but the results are not significant with either of the media used for DH induction. However, 2,4-D hormone was shows responses when used with a modified MS medium with B5 vitamins and a two-fold concentration of $CaCl_2$ for culturing lentil explants Bagheri*et al.* (2012).

The earlier data work reports in lentil from Keller and Ferrie, (2002) and Croser and Lulsdorf, (2004) showed that through microspore culture, technology, it is not possible to regenerate either Haploid or diploid plantlets. And if some induction reported up to callus levels, finding problems in determining whether the induced calli originate from gametophytic or sporophytic tissue. The absence of a robust haploid production system for and rogenesis could motivate us to finding a useful protocol through suitable culture conditions. Without the valid protocol for a particular species, the current experimental efforts to adapt Double Haploid production techniques to recalcitrant species like lentil will continue to be difficult and time consuming.

However, even under the finest situations, plant regeneration through LS and CHU (N6) medium remains difficult even after combination of different stresses like cold and centrifugation. From the current experiment, this is finding out that either of the media in various hormones (BAP and 2, 4-D) concentrations is non-responsive. In future, it opens the research approaches in different directions, this analysis could influence the choice of different media composition or same medium with different hormone stimulators in future. Alter the design of the experiment enhances the chances of getting a fruitful result. Efforts are in progress to adapt this technology to lentil to improving efficiency and integrating these techniques into routine breeding programs to accelerate genetic or breeding productivity goals.

Table 2: Linsmaier and Skoog Media (LS) Compostion

Ingredients	mg/l
Ammonium nitrate	1650.000
Calcium chloride	332.200
Magnesium sulphate	180.690
Potassium nitrate	1900.000
Potassium phosphate monobasic	170.000
Boric acid	6.200
Cobalt chloride hexahydrate	0.025
Copper sulphate pentahydrate	0.025
EDTA disodium salt dehydrate	37.300
Ferrous sulphate heptahydrate	27.800
Manganese sulphate monohydrate	16.900
Molybdic acid (sodium salt)	0.213
Potassium Iodide	0.830
Zinc sulphate heptahydrate	8.600
myo-Inositol	100.000
Thiamine hydrochloride	0.400
Sucrose	30000.000
Bavistin	30
Amphiclin	30

Table 3: CHU (N6) Media Composition

Ingredients	mg/l
Ammonium sulphate	463.000
Calcium chloride	125.340
Magnesium sulphate	90.370
Potassium nitrate	2830.000
Potassium phosphate monobasic	400.000
Boric acid	1.600
Ferrous sulphate heptahydrate	37.300
Manganese sulphate monohydrate	27.800
Potassium Iodide	3.330
EDTA disodium salt dihydrate	0.800
Zinc sulphate heptahydrate	1.500
Nicotinic acid (free acid)	0.500
Pyridoxine HCl	0.500
Thiamine hydrochloride	1.000
Glycine	2.000
Bavistin	30
Ampicillin	30

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